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Estrogen receptor stimulates target genes that have AP-1 sites with both estrogens and antiestrogens. We examined the role of ER transactivation functions (AF-1 and AF-2) in these responses. Estrogen activation requires ER transactivation functions, and may be obtained with the isolated ERα ligand binding domain. In contrast, tamoxifen activation requires the ER B region and is suppressed by AF-1. ERα truncations that delete AF-1 enhance tamoxifen action at AP-1, allow even more potent activation by raloxifene and ICI 182,780, and enhance AP1 activity in response to antiestrogens in a breast cell line. These phenotypes resemble ERβ, which naturally lacks constitutive AF-1 activity and strongly enhances AP-1 activity in the presence of antiestrogens. In every case, antiestrogen effects are independent of intact AF-2. Thus, estrogen activation at AP-1 sites involves the same transactivation functions, AF-1 and AF-2, that mediate classical response, but antiestrogen activation does not utilize these functions, and thus does not resemble conventional transactivation. We conclude that ER activates at AP-1 sites by different mechanisms with estrogens and antiestrogens, and we suggest a model of such activation.

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INTRODUCTION

Estrogen signal transduction is mediated by two related proteins, the estrogen receptors alpha (ER) and beta (ERβ) (1, 2). Both receptors are conditional transcription factors that belong to the nuclear receptor superfamily (3-5), and are comprised of separable N-terminal (AB), DNA binding (DBD) and ligand binding (LBD) domains. The ERs activate gene expression in two ways. In the best understood mode of action, or classical pathway, the ERs activate target genes by binding to specific estrogen response elements (EREs) and recruiting a p160/p300 coactivator complex to the promoter (6, 7). This coactivator complex, in turn, enhances gene expression by remodeling chromatin and, perhaps, by contacting the basal transcription machinery. In a second pathway, the ERs indirectly stimulate the expression of a different set of target genes, by modulating the transcriptional activity of heterologous transcription factors via unspecified protein-protein interactions. For example, ER enhances the activity of genes that contain AP-1 sites, the cognate binding site for the Jun/Fos complex (8-14). ER also enhances the activity of genes that contain binding sites for other transcription factors (15-18).

While the precise mechanism of ER action at AP-1 sites is unknown, our previous studies have indicated that it shows some striking differences from ER action at EREs (11, 12). First, antiestrogens, such as the breast cancer drug tamoxifen, act as potent agonists of ER action at AP-1 sites, even though they usually block ER action at classical EREs. This is particularly evident in the case of ERβ, which potently enhances AP-1 dependent transcription in the presence of antiestrogens, but not estrogen. Second, ER action at AP-1 sites does not require specific ERE recognition. ER action at AP-1 responsive reporter genes requires an AP-1 site, which does not bind ER, and AP-1 proteins, but is independent of the ER-DBD (8, 11) (R. Uht et al. manuscript submitted). Third, the strength of ER action at AP-1 sites bears little relationship to the strength of ER action at an ERE. For example, ERβ is a more potent activator of AP-1 dependent transcription than ER(α) (12), but is a weaker activator of classical estrogen response than ER(α) (19, 20).

Perhaps more surprisingly, our initial studies also suggested that tamoxifen and estrogen effects at AP-1 sites are products of completely distinct mechanisms (11). We, and others (9, 14), found that estrogen effects predominate in several breast cancer cell lines, but that tamoxifen effects predominate in other cells (including those of uterine and liver origin). Furthermore, estrogen and tamoxifen responses show different ER structure-function requirements. Estrogen activation requires the presence of the ER-LBD whereas tamoxifen activation requires the presence of the AB and DBD regions. Finally, the estrogen-liganded ER can target the VP16 transactivation function to AP-1 responsive reporters in mammalian two hybrid assays, but the tamoxifen-liganded ER cannot. This implies that the estrogen-liganded ER participates in the AP-1 protein complex, but the tamoxifen-liganded ER does not. Collectively, these results suggest that ER must participate in two sets of protein-protein interactions that lead to stimulation of AP-1 activity, one promoted by estrogen, the other promoted by tamoxifen. We now term these mechanisms the estrogen/AP-1 and antiestrogen/AP-1 pathways, respectively. ERβ, which enhances AP-1 activity in the presence of antiestrogens, bur not estrogens, only participates in the antiestrogen pathway.

It is well established that ER action at classical EREs is mediated by transactivation functions (3-5, 21, 22). ER(α) contains two separate transactivation functions, AF-1 and AF-2, which synergise strongly to give the overall level of estrogen response (23-25). While ERβ does contain a growth factor inducible AF-1 (26), its activity stems largely from AF-2 (20, 27). AF-2 consists of a hydrophobic patch which forms on the surface of the estrogen liganded LBD (28), and works by binding strongly to specific LXXLL motifs (nuclear receptor boxes) that are found within p160s (29-33). Antiestrogens, including tamoxifen, raloxifene and ICI 182,780 (ICI), prevent formation of AF-2 and thereby reduce coactivator complex recruitment (34-36). AF-1 consists of an long region of the ER(α) AB domain that binds the C-terminus of GRIP1 and other p160s (27). Even though antiestrogens bind to the ER-LBD, they are able to restrict ER(α) AF-1 activity by

promoting association of ER with corepressors (37-42). Nonetheless, tamoxifen does allow some AF-1 activity, and consequently, elicits weak agonist effects at classical EREs (21).

Because the ER transactivation functions play a central role in classical estrogen response, we ask here whether the ER transactivation functions might also play a role in ER action at AP-1 sites. We find that the estrogen/AP-1 pathway involves ER transactivation functions, but the antiestrogen/AP-1 pathway does not. Instead, the antiestrogen/AP-1 pathway is inhibited by the presence of AF-1 in ER(α). These results reinforce the notion that estrogen and antiestrogen effects at AP-1 sites are mediated by distinct mechanisms and may suggest identities for putative estrogen and antiestrogen pathway targets.

BODY

Experimental Methods

Mammalian Reporter Genes and Expression Vectors

Coll73-LUC and ERE-II-LUC have been previously described (11, 12). The ER(α) expression vectors have been previously described but, for ease of comparison, we have given ER expression vectors a consistent nomenclature. The previous names and sources of each construct are as follows:- SG5-ER=HEG0; SG5-ERV400=HE0, n101=HE302; n117=HE303; Δ129-178=HE316 (45), CMV-ER; n21=n21; n41=E41; n87=A87; n109=M109; ERΔAB (46). The coactivator expression vectors (GRIP1 and GRIP1 Δ1121C) have each been previously described (27).

The ER-LBD expression vector SG5-LBD and its mutant derivatives were constructed from pKCR2-HE14 (69), which encodes human ER-LBD amino acids 282-595. First, the LBD coding sequence was moved into pSG5 as an EcoRI fragment. A V400 mutation, which was present in the original human cDNA clone (70), was corrected by incorporating wild type human sequences from the full length pSG5-ER as a HindIII/BamHI fragment. The resulting ER LBD expression vector was subjected to point

mutagenesis with a PCR based method designed to incorporate oligonucleotides into the LBD cDNA (Stratagem). The mutations converted methionine 543 and leucine 544 to alanine residues (LBDmAF-2) or introduced a stop codon after tyrosine 537 (LBDΔAF-2). ERs bearing specific amino acid substitutions at serine 118 and at lysine 362 (ERα) or lysine 269 (ERβ) were derived by a similar methods. The nature of each mutant ER was confirmed by sequence analysis.

ERβ530 was generated from a CMV vector containing an a full length cDNA clone (Stefan Nilsson et al, unpublished). An EcoRI fragment spanning the 5' end of the longer cDNA was obtained and substituted into our existing ERβ485 expression vector (12). The orientation of the insert was confirmed by restriction analysis and sequencing. The GST-ERβ AB domain fusion protein was generated by amplification of the sequences homologous to the AB domain (amino acids 1-144) by PCR and subcloning the resulting fragment into the BamHI/EcoRI sites of pGEX-5X-3.

Cell Culture and Transfection

HeLa cells were maintained and transfected as previously described (11), except that 2μg of luciferase reporter was employed. Also included in each transfection was 1μg of pJ3 β-galactosidase control. Cell lysates were prepared thirty six to forty hours after transfection and luciferase and β-galactosidase assays were performed using standard methods described in the reference above. Where indicated, luciferase activities were corrected for variations in transfection efficiency using β-galactosidase activity.

Protein Binding Assays

Fusions of GST to various domains of the human ER were prepared as described (71). Bacteria expressing the fusion proteins were resuspended in buffer IPAB-80 (20 mM HEPES, 80 mM KCl, 6 mM MgCl2, 10% Glycerol, 1 mM DTT, 1 mM ATP, 0.2 mM PMSF and protease inhibitors; pH 7.9), sonicated mildly, and the debris was pelleted at

12,000 rpm for 1 hr in an ss34 rotor. The supernatant was incubated for 2 hrs with 500 µL of glutathione sepharose 4B beads that were previously washed with 5 volumes of PBS 0.2 % Triton X-100 and equilibrated with 5 volumes of IPAB 80. GST-fusion proteins beads were then washed with 5 volumes of PBS 0.05% Nonidet P-40 and resuspended in 1 ml of IPAB-80 for storage at 4 C until use. All the above procedures were done in a cold room at 4°C.

Assays of GST-ER fusions were carried out in 100 µL volume that contained 40 µL of bead suspension (equivalent to 10 µL of compact beads volume) and 1µL of 35S *in vitro* translated GRIP1 in IPAB-80 2.5% non fat milk and incubated for 1.5 hr at 4°C. Beads were washed 5 to 6 times with IPAB-80 containing 0.05% NP-40. Input labeled proteins, proteins bound to GST, and the ER fusion beads were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide and then to autoradiography.

Results And Discussion

The Estrogen/AP-1 Pathway Requires Only the LBD and AF-2

To understand how ER enhances AP-1 activity, we asked whether either of the conventional ER transactivation functions plays a role in ER action at AP-1 sites. To perform these experiments, we asked whether transiently transfected wild type ERs, or ER derivatives bearing specific mutations in AF-1 or AF-2, would elicit estrogen or tamoxifen responses from an AP-1 responsive reporter in HeLa cells. We previously established that estrogen effects could be obtained at AP-1 sites in the absence of either the ER N-terminal domain or DBD, suggesting that the LBD is necessary for the estrogen/AP-1 pathway (11). We therefore first asked whether the LBD was also sufficient to obtain an estrogen response at AP-1 sites. Fig. 1 shows that wild type ER-LBD gave some weak constitutive activation of the AP-1 responsive reporter gene relative to empty expression vector. Addition of estrogen elicited a further ten fold increase in the activity of the AP-1

responsive promoter. Tamoxifen (shown) and other antiestrogens (not shown) failed to enhance AP-1 activity in the presence of the isolated LBD.

We then asked whether the LBD mediated estrogen response required AF-2. ER-LBDs bearing either a specific mutation in AF-2 (LBDmAF-2; M543A.L544A), or a complete truncation of ER helix 12 (LBDΔAF-2, truncated at position 537) gave weak constitutive activation at the AP-1 site, but failed to yield further estrogen activation. Similar requirements for AF-2 were also noted in another ER mutant that elicits estrogen, but not tamoxifen, responses at AP-1 sites (ERK206A; R. Uht et al. manuscript submitted). We conclude that AF-2 plays a key role in the estrogen/AP-1 pathway. Because AF-2 works by binding p160 coactivators, this result may also suggest that the estrogen/AP-1 pathway involves ER/coactivator contacts.

AF-1 Suppresses Tamoxifen Effects at AP-1 Sites

We next turned our attention to AF-1. In Hela cells, full AF-1 activity requires an extended region of the AB domain (amino acids 41-129, Fig. 2) (27, 43-46). In accordance with our previously published results (27), several ERs bearing N-terminal deletions which eliminate AF-1 activity elicited no tamoxifen response, and reduced estrogen response, at the classical ERE (N101, N109, N117, ΔAB; Fig. 2B). Moreover, an ER internal deletion which retains AF-1, but lacks sequences in the B region between amino acid 129 and the DBD (Δ129-178), elicited normal activity in the presence of both ligands, and increased ligand independent activity.

In sharp contrast to the phenotypes that were observed at the classical ERE, ERs lacking the first 101 and 109 amino acids of the AB domain (N101, N109) showed accentuated tamoxifen responses at the AP-1 responsive reporter gene (Fig. 2A). This result indicates that tamoxifen responses at AP-1 sites are independent of AF-1 activity *per se* and may actually be suppressed by AF-1. ERs with longer N-terminal deletions (N117 or ERΔAB) and the ER lacking the B region (Δ129-178) each failed to elicit tamoxifen

responses from the AP-1 responsive reporter. Thus, tamoxifen activation at AP-1 sites requires the proximal (B) region of the ER N-terminal (AB) domain. Estrogen responses at the AP-1 site were either unaffected or modestly reduced by the N-terminal deletions (N101, N109, N117, ΔAB) and unaffected by deletion of the B region (ERΔ129-178). Thus, estrogen responses that are obtained in the presence of full length ER at AP-1 sites are independent of the ER B region, but may require a small contribution from AF-1.

Serine 118 is Dispensable For Tamoxifen Effects at AP-1 Sites

Tamoxifen activation at AP-1 sites requires an unspecified function whose N-terminal boundary lies between amino acids 109 and 117 (see Fig. 2). Full AF-1 activity requires a key serine residue (S118), that is a target for MAP kinase phosphorylation and forms part of a MAP kinase consensus recognition sequence (PXXSP) that lies within this region (47-52). We therefore asked whether S118 played any role in ER action at AP-1 sites. We utilized ER mutants, in which S118 was mutated to either alanine (A), arginine (R), which has a bulky charged side chain and should disrupt S118 dependent protein-protein contacts, or glutamic acid (E), which mimics an active phosphorylated serine residue (51).

Figure 3A reveals that each ER S118 mutant elicited tamoxifen responses at AP-1 sites that were comparable to those obtained with wild type ER. Similar results (not shown) were obtained with an S118A mutation in the context of the isolated ER AB-DBD region, which constitutively enhances gene expression in a manner that resembles the tamoxifen-liganded ER (11, 53). Thus, serine 118 is not essential for tamoxifen activation at AP-1 sites. Control transfections showed that ER-S118E gave enhanced tamoxifen response relative to wild type ER and the S118A and S118R mutants at the classical ERE (Fig. 3B). This agrees with the notion that S118E mimics the phosphorylated, active, state of AF-1 and that S118A and S118R block AF-1 phosphorylation and do not allow full AF-1 activity. Because the S118E mutation did not give significantly increased tamoxifen

response at the AP-1 site, this result reinforces the notion that tamoxifen activation at AP-1 sites does not reflect conventional AF-1 activity.

Removal of ER(α) AF-1 Creates a Phenotype That Resembles ERβ

Removal of ER(α) AF-1 allows ER to elicit accentuated tamoxifen responses at AP-1 sites (Fig. 2). This phenotype is similar to ERβ, which naturally lacks a constitutive AF-1 activity and elicits strong tamoxifen responses at AP-1 sites (12). We therefore next asked whether removal of ER(α) AF-1 created other ERβ-like phenotypes.

We had previously shown that full length ER(α) enhances AP-1 activity in the presence of tamoxifen, but not raloxifene, but ERβ strongly enhances AP-1 activity in the presence of a range of antiestrogens (12). Accordingly, wild type ER(α) enhanced AP-1 activity in the presence of tamoxifen, estrogen and the synthetic estrogen agonist, diethylstilbestrol (DES), but not in the presence of ICI or raloxifene (Fig. 4A). Two ERs bearing truncations of 21 or 41 amino acids from the ER amino-terminus (N21, N41), which both retain full AF-1 activity, gave a similar profile of ligand activity. However, an ER bearing a truncation of 87 amino acids (N87), which shows reduced AF-1 activity (27, 46), gave slightly enhanced tamoxifen activation at the AP-1 site. More dramatically, an ER bearing a further truncation to position (N109), which totally eliminates AF-1 activity (27, 46), gave large ICI and raloxifene responses at the AP-1 site. Intriguingly, an ER bearing a complete truncation of the AB domain (ΔAB) remained able to elicit strong raloxifene and ICI responses at the AP-1 site, even though it was unable to mount a tamoxifen response (see Conclusions). However, in general, the tamoxifen, raloxifene and ICI effects obtained with the ER truncations were comparable to those obtained with ERβ (ERβ485). We conclude that AF-1 suppresses the ability of several antiestrogens to enhance AP-1 responsive transcription.

We also previously established that full length ER(α) shows little activity at AP-1 sites in breast cells, and that ERβ elicits strong antiestrogen responses at AP-1 sites in the

same cell types (12). Fig. 4B shows that an ER(α) AF-1 mutant (N109), elicited extremely potent ICI and raloxifene effects and more modest tamoxifen effects from the AP-1 responsive reporter in MCF-7 cells. In parallel, ERβ elicited strong ICI, raloxifene and tamoxifen responses at the AP-1 site. However, ER(α) only elicited very weak responses in the presence of estradiol and DES. Thus, removal of AF-1 allows the antiestrogen liganded ER to strongly enhance AP-1 activity in MCF-7 cells. Taken together, our results suggest that removal of ER(α) AF-1 allows ER(α) to act like ERβ at AP-1 sites.

Tamoxifen Activation at AP-1 Sites is Independent of the AF-1 Binding Region in GRIP1

ER AF-1 binds the C-terminal region of GRIP1. We previously showed that a GRIP1 truncation (GRIP1Δ1121C), which lacks the AF-1 binding region, acts as a specific dominant negative for AF-1 activity (27). Because our results indicate that AF-1 is dispensable for tamoxifen responses at AP-1 sites, we asked whether blocking AF-1 activity with this dominant negative GRIP1 would affect ER action at AP-1 sites.

Fig. 5 reveals that wild type GRIP1 enhanced basal AP-1 activity. In contrast, the GRIP1 dominant negative failed to enhance basal AP-1 activity, indicating that the GRIP1 C-terminus contains functions that are important for GRIP1 action at AP-1 sites. However, while both tamoxifen and estrogen enhanced AP-1 activity in the presence of wild type GRIP1, only tamoxifen enhanced AP-1 activity in the presence of dominant negative GRIP1. Thus, blocking AF-1 activity reduces estrogen response at AP-1 sites, but accentuates tamoxifen response. This result is consistent with the notion that the full length ER does not require AF-1 to participate in the antiestrogen/AP-1 pathway.

Different ERβ isoforms Lack AF-1 Activity

Native ERβ protein possesses at least two N-termini. One form (61Kd), is 530 amino acids long and is produced by translation of the complete ERβ open reading frame (ERβ530) (54, 55). A second form (55Kd), lacks, approximately, the first 45 amino acids of the longer form and is similar in length to a cDNA clone that has been previously characterized (ERβ485) (56). We previously showed that the shorter form (ERβ485) lacks constitutive AF-1 activity and strongly enhances AP-1 activity (12, 27). In the light of the results described above, we next asked whether the long form of ERβ might contain an AF-1 activity that would suppress its ability to enhance AP-1 responsive transcription.

Fig. 6A indicates that both ERβ530 and ERβ485 failed to elicit tamoxifen responses at the classical ERE, even in the presence of high levels of GRIP1 and p300. ER(α), however, elicited extremely potent AF-1 dependent tamoxifen responses under similar conditions (Lower panel, note expanded scale). Further experiments also revealed that both forms of ERβ gave a comparable degree of activation at the ERE responsive reporter across a wide range of levels of transfected ER and failed to show agonist activity in the presence of tamoxifen, raloxifene or ICI (not shown). We previously showed that the ER(α) AB domain contains a constitutive binding site for GRIP1, but that the shorter form of the ERβ does not (27). We therefore generated a GST fusion protein containing the entire AB domain of the long form of ERβ (amino acids 1-144) and asked whether it would bind GRIP1 (Fig. 6B). While the ER(α) AB domain bound well to GRIP1, the ERβ AB domain did not. Thus, the AB domain of the ERβ long form lacks a constitutive GRIP1 binding site.

We then asked whether the longer form of ERβ would enhance AP-1 responsive transcription. Figure 6C shows that both forms of ERβ showed comparable abilities to enhance AP-1 responsive transcription, with the longer form (ERβ530) actually yielding larger antiestrogen effects than the shorter form (ERβ485). Thus, both the long and short forms of ERβ lack constitutive AF-1 activity and a constitutive GRIP1 binding region in

their AB domains. Both forms also strongly enhance AP-1 responsive transcription in the presence of antiestrogens.

AF-2 is Not Needed For Antiestrogen Action at AP-1 Sites

Finally, we examined the role of AF-2 in antiestrogen response at AP-1 sites. AF-2 consists of a hydrophobic cleft, made up of a cluster of residues from LBD helices 3, 5, 6 and 12, that forms upon the surface of the holo-ER (28, 34, 35). We utilized a mutation in a helix 3 residue (K>A) (57), which forms key hydrogen bonds with the GRIP1 nuclear receptor box, thereby helping ER AF-2 to specifically interact with the p160 coactivator (35).

We first examined whether the mutation of AF-2 would affect the ability of ER(α) to elicit antiestrogen responses at AP-1 sites in HeLa cells. Figure 7A shows that the AF-2 mutant (K362A) gave reduced estrogen response and wild type levels of tamoxifen response at AP-1 sites. The equivalent ERβ AF-2 mutant (K269A) gave ICI, raloxifene and tamoxifen responses that were comparable to, or slightly larger than, wild type receptor in Hela cells (Fig. 7B), and ER(α) K362A mutation also gave modestly enhanced raloxifene and ICI responses in the context of the DBD-LBD region at AP-1 sites (not shown). Similar results were obtained in MCF-7 cells (not shown). Control transfections showed that the AF-2 mutant receptors were inactive at ERE responsive reporters (not shown). Thus, intact AF-2 is not required for ER action in the antiestrogen/AP-1 pathway and AF-1 plays a more prominent role in suppressing antiestrogen effects at AP-1 sites (See CONCLUSIONS).

Recommedations in relation to the Statement of Work

This report covers some of the same material as found in a previous report (1996), but goes far deeper into the mechanisms of action of ER at AP-1. In particular neither the original grant application nor the statement of work anticipated that a new estrogen receptor, ERb, would be discovered, and would be found to have a potent ability to activate AP-1 target

genes with antiestrogens (12). This discovery has altered somewhat the goals of the work, since it is now of prime importance to understand the differences between ERa and ERb action at AP-1. In this report we trace the difference to the absence of a conventional AF-1 in ERb.

This is the final report. Looking back at the statement of work, we feel very satisfied about our success and the importance of our discoveries especially those related to Task 1 (Analysis of the mechanism of antiestrogen agonism at AP-1 sites). In addition to this report, they have been described in published papers (11, 12). We also feel we have had significant success with Task 2, 3, and 5 (11,13). Task 4 ,testing whether the AP-1 pathway of estrogen response is more active in antiestrogen resistant breast tumor cell lines, has been the mostly difficult to make progress. We obtained several derivatives of MCF-7 cells that were described in the literature as antiestrogen resistant, and did observe some differences from the parental cells in response at AP-1. However, the differences varied with growth conditions and were difficult to reproduce. Furthermore, we were unable to confirm that the cell lines displayed a consistent growth response to antitestrogens in our lab. Thus task 4 may need to await the development of cell lines with a more dramatic growth response to antiestrogens. As noted above, with the exception of task 4, all the other tasks have led to published results, including papers in Molecular Endocrinology, Endocrinology, and Science. We hope we can do as well in all our research plans.

CONCLUSIONS

ER Enhances AP-1 Activity Via Two Distinct Mechanisms With Different Requirements For Transactivation Functions

Several lines of evidence suggest that the ERs possesses the capacity to modulate gene expression via mechanism that is stimulated by antiestrogens and not estrogens. While, estrogen and tamoxifen both enhance AP-1 activity in the presence of ER(α), estrogen and

tamoxifen responses at AP-1 sites showed different characteristics suggesting that they are actually the products of completely distinct mechanisms (11). We originally called these mechanisms the β (estrogen) and α (antiestrogen) pathways, but we now refer to them as the estrogen and antiestrogen pathways to avoid confusion with the different ER isoforms. Later, we showed that ERβ does not enhance AP-1 dependent transcription in the presence of estrogens, but does strongly enhance AP-1 dependent transcription in the presence of antiestrogens (12), suggesting that ERβ participates exclusively in the antiestrogen/AP-1 pathway. Furthermore, endogenous genes have now been described that respond weakly to estrogens, and strongly to antiestrogens (15, 17, 18).

The results in this paper strongly reinforce the notion that ER activates transcription via an antiestrogen specific mechanism. We find that estrogen effects at AP-1 sites require only the isolated ER-LBD. In contrast, tamoxifen effects require an intact B region and, as shown by studies in an accompanying paper, an intact DBD and hinge (R. Uht et al, manuscript submitted). Thus, ER utilizes different surfaces to elicit estrogen and tamoxifen effects at AP-1 sites. We also find that estrogen activation involves AF-2 in the context of the ER-LBD (Fig.1), or AF-1 and AF-2 (Figs. 3, 7B), in the context of full length ER. Tamoxifen activation is independent of both ER activation functions, and, indeed, is strongly suppressed by AF-1. Other antiestrogen effects (tamoxifen, raloxifene and ICI responses obtained with ERβ and raloxifene and ICI responses obtained with the ER(α) DBD-LBD region) are also independent of ER transactivation functions. Estrogen and tamoxifen responses at AP-1 sites can be further distinguished by their requirement for the C-terminal AF-1 binding region of the p160 coactivator, GRIP1 (Fig. 5). Thus, estrogen and antiestrogen effects at AP-1 sites occur via distinct mechanisms. The estrogen/AP-1 pathway requires ER transactivation functions and the antiestrogen/AP-1 pathway utilizes a separate mechanism that does not resemble conventional transactivation.

A Model For Estrogen and Antiestrogen Effects at AP-1 Sites:-A Role For Coactivator/Corepressor Recognition?

We have previously shown that the estrogen/AP-1 and antiestrogen/AP-1 pathways are mediated by protein-protein interactions (11). What are the protein targets of the two pathways?

Because the estrogen/AP-1 pathway requires ER transactivation functions, and the ER transactivation functions bind p160 coactivators, we propose that the estrogen/AP-1 pathway involves ER contact with p160s. In classical estrogen response, ER/coactivator contacts serve to bring the coactivator complex to target promoters. We speculate that ER/coactivator interactions do not play the same role the ER/AP-1 pathway. We know that the isolated ER-LBD strongly enhances AP-1 responsive transcription in an AF-2 dependent manner (Fig. 1), even though it does not bind jun or fos (11). We also know that AP-1 proteins activate transcription by recruiting a CBP-p160 complex via direct contacts with CBP (30). Thus, ER/coactivator interactions could serve to bring ER to the AP-1 site, via contacts with p160s that are associated with CBP in the AP-1 coactivator complex (Fig. 8A). We are presently investigating this issue by asking whether ER enhances the activity of isolated coactivator proteins (P. Webb et al, manuscript in preparation).

The antiestrogen/AP-1 pathway does not involve ER transactivation functions. Our studies do not directly address the antiestrogen pathway target, and, in principle, tamoxifen-liganded ER could enhance AP-1 activity at many different levels. Nonetheless, it is attractive to speculate that the antiestrogen liganded ER might enhance AP-1 activity by binding and sequestering a corepressor that is present in the AP-1 complex (Fig. 8B). Several pieces of evidence are consistent with this notion. First, mammalian two hybrid assays indicate that the tamoxifen liganded ER does not directly participate in the AP-1 complex in vivo, suggesting that it works away from the AP-1 responsive promoter (11). Second, tamoxifen effects at AP-1 sites are mediated by a tamoxifen-specific pathway, and

corepressors are known to preferentially associate with steroid receptor/anti-hormone complexes, rather than receptor/hormone complexes (40, 58). Third, our own results indicate that the ER(α) B region is essential for tamoxifen responses at AP-1 sites, and that the B region represses ER action at classical EREs in the presence of cotransfected GRIP1 (27). We are presently investigating whether ER action at AP-1 sites involves any of the known corepressors, such as N-COR and SMRT.

Our model also suggests an explanation for the ability of ER AF-1 to suppress antiestrogen effects at AP-1 sites. If the estrogen/AP-1 pathway represents binding to coactivators that are present at the promoter and the antiestrogen/AP-1 pathway represents sequestration of corepressors away from the promoter then these pathways must be mutually exclusive. It has also been suggested that nuclear receptors do not physically interact with coactivators and corepressors simultaneously (6, 58), underlining the mutual exclusivity of the putative estrogen and antiestrogen pathways. Thus, when ER is liganded to estrogens, the ER transactivation functions would connect ER to the coactivator complex at the AP-1 site and block the ability of ER to sequester corepressors away from the AP-1 site. Antiestrogens (which prevent AF-2/p160 interactions) and removal of AF-1 would be required before ER(α) would be free to bind and sequester corepressors. Because ERβ lacks AF-1, addition of antiestrogens would be sufficient to allow ERβ to bind and sequester corepressors.

Different Antiestrogens Utilize Different Surfaces Of ER(α) To Enhance AP-1 Activity

Raloxifene strongly enhances AP-1 activity in the presence of the isolated ER DBD-LBD region, yet tamoxifen does not (Fig. 4B). This indicates that there are functional differences between the raloxifene and tamoxifen-liganded ER(α) LBDs. These unspecified differences may impact upon LBD/AB domain cross-talk. We know that full length tamoxifen liganded ER(α) enhances AP-1 activity, but the raloxifene liganded

ER(α) does not (12). We also show here that AF-1 partially suppresses the ability of ER(α) to enhance AP-1 responsive transcription in the presence of tamoxifen, but completely suppresses the ability of ER(α) to enhance AP-1 responsive transcription in the presence of raloxifene (Fig.4A). Similar complexities in LBD/AB domain cross-talk have been observed at classical EREs. Antiestrogens all permit ER to bind DNA (59). Nonetheless, while raloxifene and ICI allow little AF-1 activity (60, 61), tamoxifen allows high AF-1 activity (21). Thus, antiestrogens exert distinct effects upon LBD/AB domain cross-talk in diverse gene regulation assays. On the basis of our model (Fig. 8), we expect that these effects stem from complexities in the way that corepressors interact with ER in the presence of different antiestrogens.

Antiestrogens Enhance Gene Expression By Two Mechanisms With Opposite Requirements For AF-1

It is important to understand the molecular mechanisms of antiestrogen agonism. Antiestrogens display a characteristic spectrum of mixed estrogen-like agonist effects in patients (62, 63). While antiestrogens all block growth of early stage breast cancers, tamoxifen stimulates uterine growth, increases the risk of uterine cancer, and often switches from inhibition to stimulation of breast cancer growth during tumor progression. Tamoxifen also elicits desirable estrogen-like effects in other tissues, such as blocking bone loss and protecting against heart disease. Raloxifene blocks estrogen action in uterus (64), but retains desirable estrogen-like effects upon bone and the cardiovascular system (65, 66). ICI acts largely as a pure antiestrogen (67), but shows some agonist effects in bone (68). Another ER ligand, GW5638 displays minimal uterotrophic activity, but acts as full ER agonist in bone and the cardiovascular system (61). Understanding the source of these antiestrogen agonist effects will help in the development of breast cancer drugs and hormone replacement therapies with more favorable profiles of action.

Antiestrogens block AF-2 activity (34, 35). Nonetheless, tamoxifen leaves ER(α) AF-1 activity unaffected (21). ER(α) AF-1 is therefore one potential source of antiestrogen agonist effects. Antiestrogens also induce gene expression by modulating the transcriptional activity of heterologous transcription factors (11, 12, 15-18). We show here that tamoxifen, raloxifene and ICI effects at AP-1 sites are suppressed by AF-1. Thus, antiestrogens enhance gene expression via, at least, two completely distinct mechanisms with opposite requirements for AF-1. An informed strategy to block or promote specific antiestrogen effects in vivo will require an understanding of the relative contributions of each mechanism to particular antiestrogen effects in vivo.

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FIGURE LEGENDS

Fig. 1. The Estrogen Pathway Requires only the ER(α)-LBD and AF-2. Results of a HeLa cell transfection in which we monitor the effect of ligands upon the activity of a transiently transfected AP-1 responsive reporter in the presence of empty expression vector or expression vectors for the isolated ER-LBD (amino acids 282-595), or similar LBDs bearing mutations in AF-2. The mutant LBDs contained a double hydrophobic mutation in AF-2 (LBDmAF-2; M543A,L544A) or a complete truncation of helix 12 and F domain sequences downstream of amino acid 537 (LBDΔAF-2). The individual bars in each graph represent luciferase activity in extracts of cells treated with ethanolic vehicle (open bars), 5μM tamoxifen (gray bars) or 10nM estradiol (black bars). The bars show average luciferase activities calculated from triplicate wells in a representative transfection.

Fig. 2 AF-1 Suppresses Tamoxifen Effects at AP-1 sites. (A) The behavior of deletions that eliminate different portions of the ER N-terminal (AB) domain at AP-1 sites. A schematic of ER truncations and N-terminal (AB) Domain Structure. The extent of sequences required for full AF-1 activity in HeLa cells is marked in gray. The effect of ER ligands upon the activity of a transiently transfected coll73-LUC reporter gene in HeLa cells is shown in the accompanying graph. Also included in the transfection were an empty SV40 expression vector, SG5 (none) or SV40 expression vectors for wild type ER, or ERs lacking the first.101 (N101), 109 (N109); 117 (N117) amino acids of the AB domain, an ER lacking the entire AB domain (ΔAB), or an ER containing an internal deletion of ER sequences 129-178 (Δ129-178). The experiment was carried out as described in Fig.1. The individual points show average fold inductions, relative to the no hormone treatment, calculated from triplicate wells in a representative transfection. (B) The behavior of the same deletions at an ERE responsive reporter gene, EREII-LUC. The experiments were

carried out in a similar manner to Fig. 2A. The individual points show luciferase activities, calculated similarly to Figure 1B.

Fig. 3. Serine 118 is Dispensable For ER Action at AP-1 sites. (A) The effect of three point mutations in phosphorylation target residue serine 118 on ER action at AP-1 sites. S118A= Alanine; S118R= Arginine; S118E= Glutamic Acid. One typical experiment is shown. (B) Experiment as in Fig. 2A, except that the EREII-LUC reporter is used. The figure represents the average of four separate experiments. Each point was normalized to the activity of the reporter gene in the absence of ER and ligand.

Fig. 4. AF-1 Suppresses Antiestrogen Action at AP-1 Sites. (A) AF-1 suppresses the capacity of ER(α) to enhance AP-1 activity in the presence of antiestrogens. The effect of various ER ligands upon the activity of a transiently transfected coll73-LUC reporter gene was examined in HeLa cells. Also included in the transfection were an empty CMV expression vector (none) or CMV expression vectors for wild type ER, ERs lacking different portions of the ER N-terminus, or a CMV expression vector for human ERβ485. The ligands were ICI 182,780 (10nM); raloxifene (10nM), tamoxifen (5μM), estradiol (10nM) or DES (10nM) (B) Removal of AF-1 Changes the Ligand Preference of ER(α) action in MCF-7 Cells. Results of a series of transfections, carried out in MCF-7 breast cells. A schematic of the SV40 driven expression vectors for each receptor is shown at left. The ligands were as described in Figure 4A. Fold inductions represent the amount of luciferase activity obtained in the presence of ligand over that obtained with vehicle alone.

Fig. 5. Tamoxifen Effects at AP-1 sites are Independent of The AF-1 Recognition Site on GRIP1. The effect of ligands upon the coll73-LUC reporter gene was examined in the presence of an ERV400 expression vector and SV40 driven expression vectors for wild

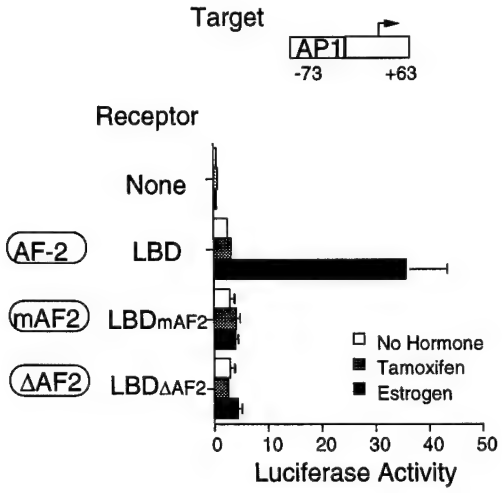
type GRIP1 or a GRIP1 truncation that is incapable of binding AF-1. A schematic of GRIP1, indicating the approximate position of AF-1 binding in gray, is shown at the left.

Fig. 6. ERβ Isoforms Lack AF-1 Activity and Enhance AP-1 Activity in the Presence of Antiestrogens. (A) Overexpression of GRIP1 and p300 fail to elicit tamoxifen responses at classical EREs in Hela cells in the presence of either form of ERβ. Effects of a combination of GRIP1 and p300 (CoAcs) on EREII-LUC expression in the presence of SV40 driven expression vectors for human ERβ, or ER(α). Note the expanded scale for the transfection containing ER(α). (B) Autoradiogram of an SDS-PAGE gel showing labeled GRIP1 protein retained by various GST-fusion proteins. The lanes represent consist of 10% input GRIP1, then GRIP1 protein retained by GST- beads, GST-ERβ AB domain fusion, GST-ER(α) AB domain, GST-ER(α) LBD and GST-ER(α) LBD in the presence of estrogen. (C) Effect of ERβ530 and ERβ485 upon AP-1 responsive transcription in HeLa cells. A range of ligands was utilized as in Fig. 4.

Fig. 7. Mutation of AF-2 Does Not Affect Antiestrogen Action At AP-1 sites. (A) Effect of tamoxifen or estradiol upon AP-1 responsive transcription in the presence of either full length ER(α), or the AF-2 mutant (K362A). The data is shown as fold increase of luciferase activity in the presence of ligand over no hormone. (B) Effects of different ligands upon AP-1 responsive transcription in HeLa cells in the presence of full length ERβ or the ERβ AF-2 mutant (K269A).

Fig. 8. Models for the Two Pathways of ER Action at AP-1 sites. (A) The Estrogen/AP-1 Pathway. Estrogen-liganded ER uses its own transactivation functions (AF-1 and AF-2) to recognize p160s that are present in the AP-1 complex. From this location, ER enhances AP-1 dependent transcription. (B) The Antiestrogen/AP-1 Pathway. Antiestrogen liganded ER binds and sequesters repressors that are present in the

AP-1 complex through regions of ER that do not interact with coactivators. AP-1 activity is thereby enhanced by ERs that do not directly participate in the AP-1 complex.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Receptor
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LBD
mAF2
LBDmAF2
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Estrogen
0
10
20
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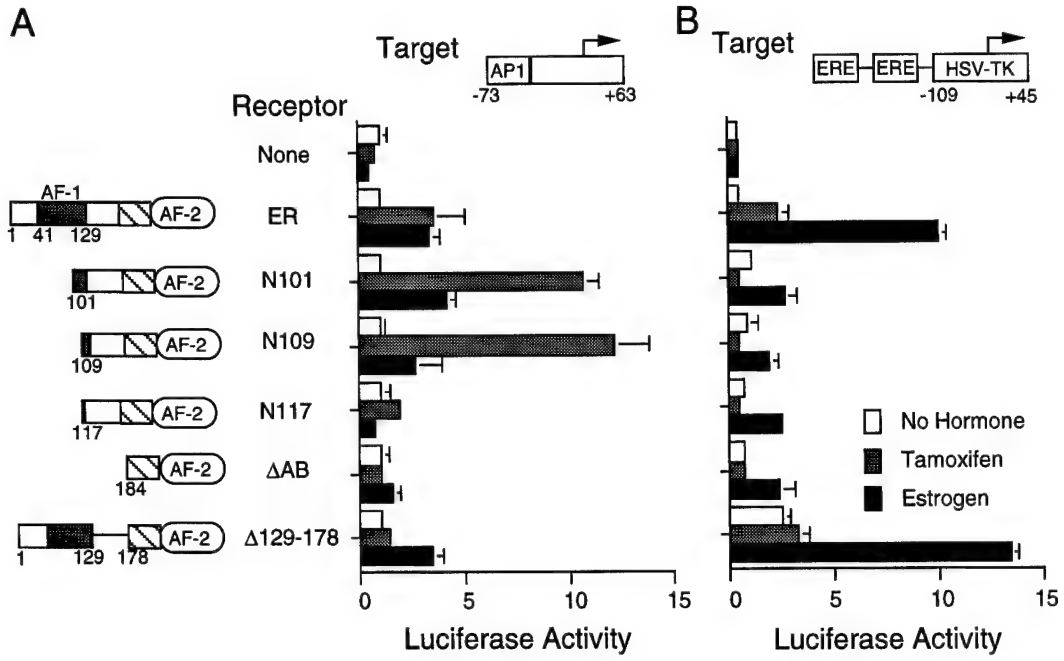
Luciferase Activity

Fig. 1.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AP1
-73
+63
Receptor
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N117
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Δ129-178
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AF-2
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129
101
109
117
184
178
0
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15
Luciferase Activity
B
Target
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-109
+45
No Hormone
Tamoxifen
Estrogen
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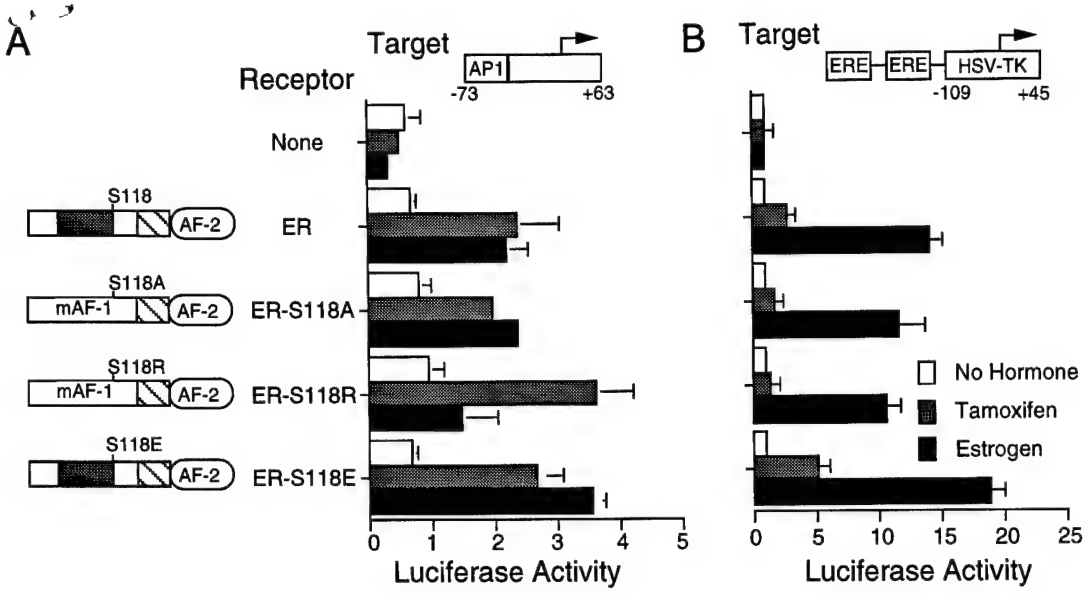
Luciferase Activity

Fig. 2.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Target
Receptor
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+63
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mAF-1
AF-2
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S118E
AF-2
ER-S118E
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1
2
3
4
5
Luciferase Activity
B
Target
ERE
ERE
HSV-TK
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+45
No Hormone
Tamoxifen
Estrogen
0
5
10
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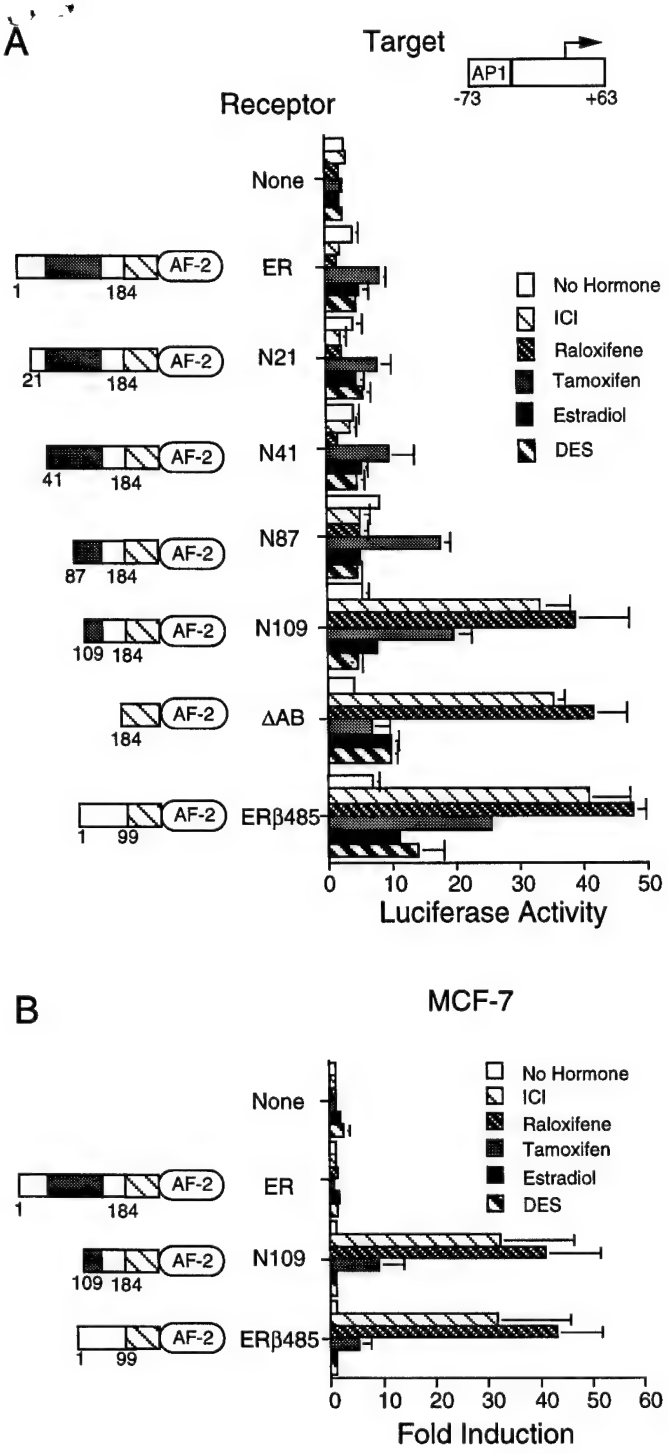
20
25
Luciferase Activity

Fig. 3.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Target
AP1
-73
+63
Receptor
None
ER
N21
N41
N87
N109
ΔAB
ERβ485
AF-2
1
184
21
41
87
109
99
No Hormone
ICI
Raloxifene
Tamoxifen
Estradiol
DES
0
10
20
30
40
50
Luciferase Activity
B
MCF-7
60
Fold Induction

Fig. 4.

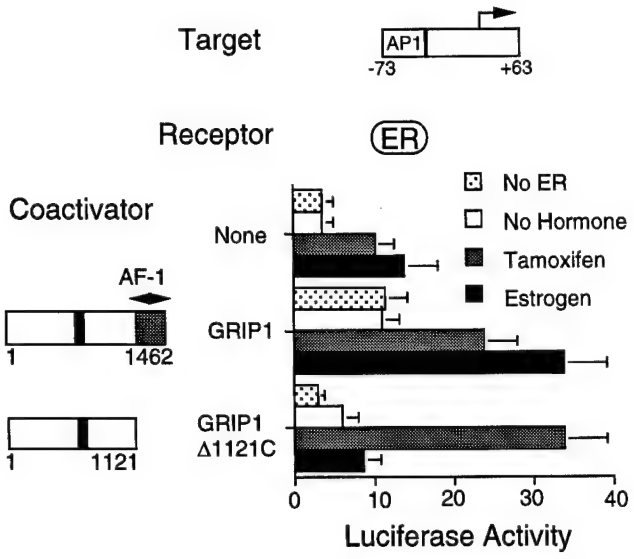

Target
AP1
-73
+63
Receptor
ER
Coactivator
AF-1
1
1462
1
1121
None
GRIP1
GRIP1
Δ1121C
No ER
No Hormone
Tamoxifen
Estrogen
0
10
20
30
40
Luciferase Activity

Fig. 5.

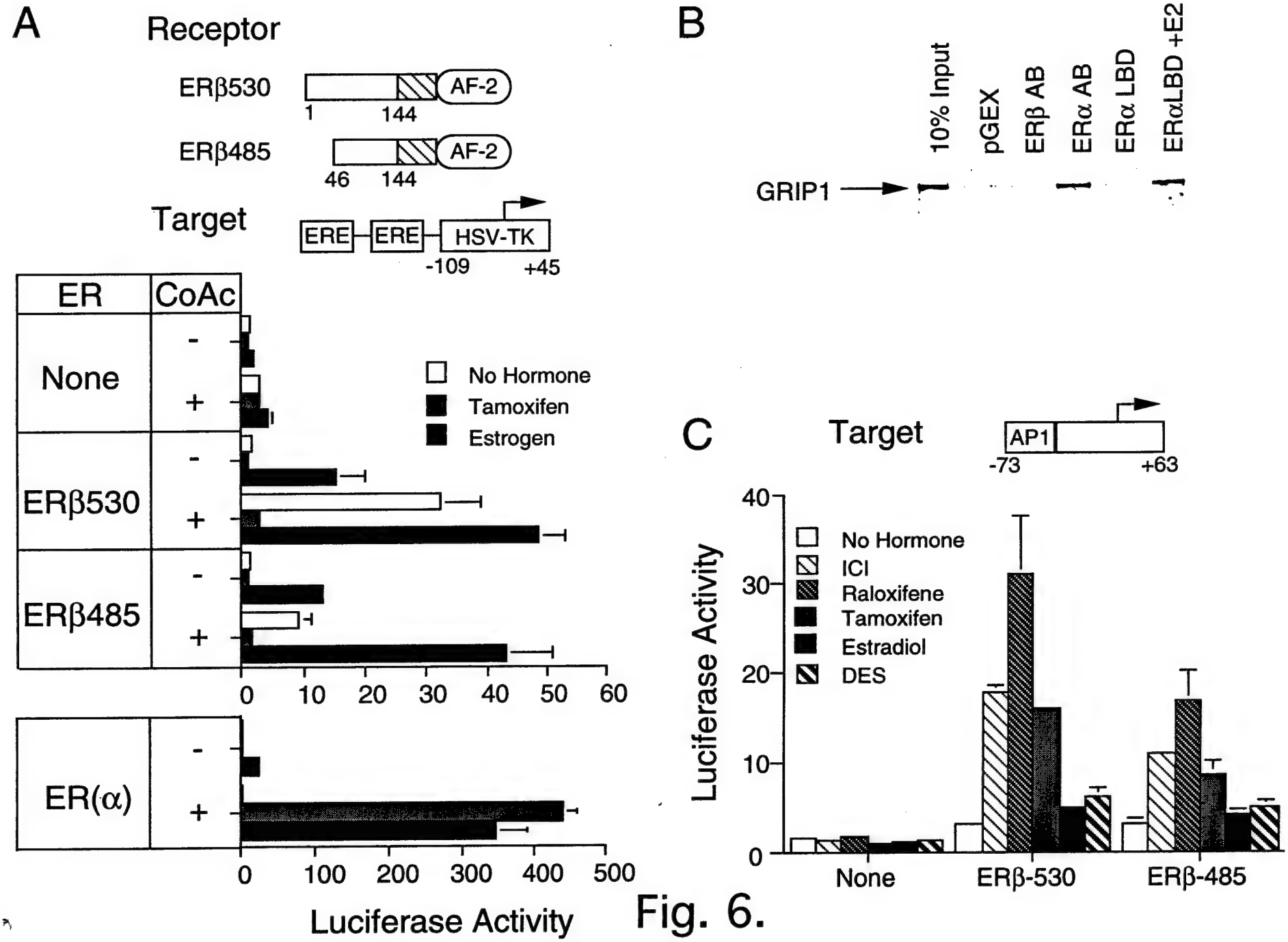

A
Receptor
ERβ530
AF-2
1
144
ERβ485
AF-2
46
144
Target
ERE
ERE
HSV-TK
-109
+45
ER
CoAc
None
ERβ530
ERβ485
ER(α)
-
+
No Hormone
Tamoxifen
Estrogen
0
10
20
30
40
50
60
0
100
200
300
400
500
Luciferase Activity
B
10% Input
pGEX
ERβ AB
ERα AB
ERα LBD
ERαLBD +E2
GRIP1
C
Target
AP1
-73
+63
Luciferase Activity
40
30
20
10
0
No Hormone
ICI
Raloxifene
Tamoxifen
Estradiol
DES
None
ERβ-530
ERβ-485

Fig. 6.

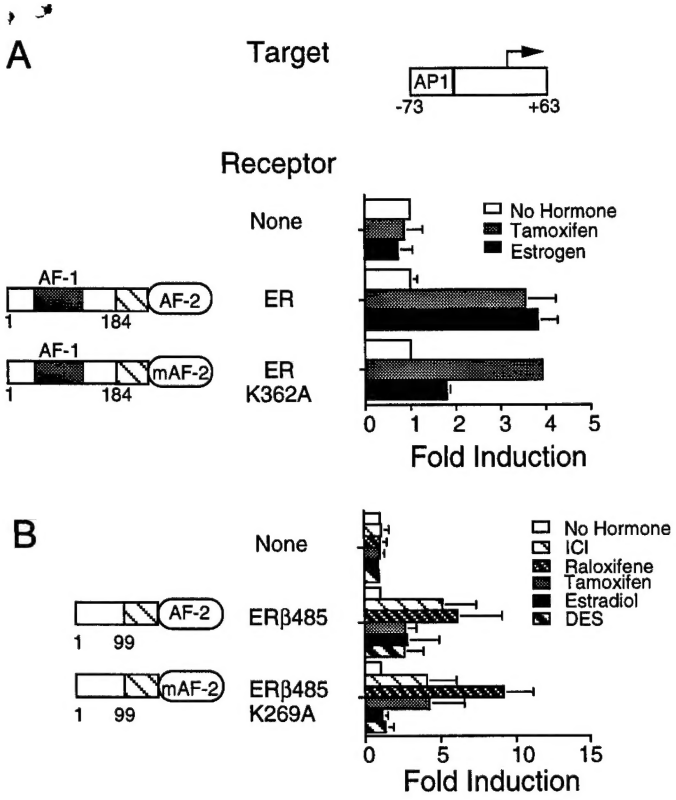
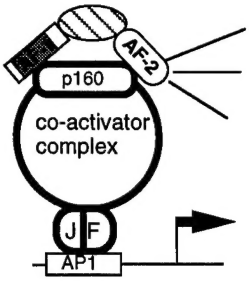

A
Target
AP1
-73
+63
Receptor
None
ER
ER
K362A
AF-1
AF-2
mAF-2
1
184
No Hormone
Tamoxifen
Estrogen
0 1 2 3 4 5
Fold Induction
B
None
ERβ485
ERβ485
K269A
AF-2
mAF-2
1
99
No Hormone
ICI
Raloxifene
Tamoxifen
Estradiol
DES
0 5 10 15
Fold Induction

Fig. 7.

A Estrogen Pathway



B Antiestrogen Pathway

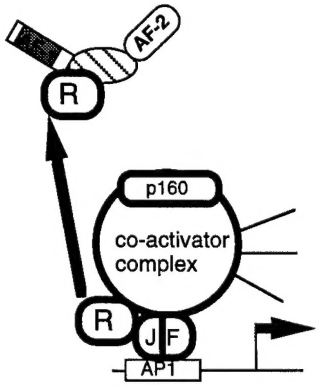


Fig. 8.

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